From the Department of Microbiology, Tumour and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

Crimean-Congo Haemorrhagic Fever Virus

Studies on Molecular Pathogenesis and Host-Cell Interactions

Helen Karlberg

Karolinska Institutet

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Crimean-Congo Hemorrhagic Fever Virus

Studies on Molecular Pathogenesis and Host- Cell Interactions

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By

Helen Karlberg

Principal Supervisor:
Associate Professor
Ali Mirazimi
Karolinska Institute
Dept. of Microbiology, tumor- and cell biology

Co-supervisor(s):
Associate Professor
Yee-Joo Tan
National University of Singapore
Dept. of Microbiology

Professor
Karl-Eric Magnusson
Linköping University
Dept. of Clinical and experimental medicin

Opponent:
Professor Roger Hewson
Public Health England
Microbiology research

Examination Board:
Professor Michael Lindberg
Linnaeus University
Dept. of Chemistry and biomedical science

Professor Sigvard Olsson
University of Gothenburg
Dept. of Clinical virology

Professor Anna Karlsson
Karolinska Institute
Dept. of Clinical microbiology

ABSTRACT

Crimean-Congo haemorrhagic fever (CCHF) disease in humans shows a spectrum of severity, from mild to acute. The pathogenesis of this and other viral haemorrhagic fevers (VHF) is associated with alteration of vascular barrier function and haemorrhage, but the specific underlying mechanism is unknown. This thesis examines the progression of the disease, in particular virus-host cell interactions and how CCHFV modulates the function of both Type I IFN response and apoptotic pathways. Programmed cell death and regulation of apoptosis in response to viral infection is an important factor for host or virus survival. In order to establish viral infection and to keep the virus contagious, it is also important to evade antiviral responses such as Type I IFN response.

An initial study showed that replicating CCHFV delays the Type I IFN response, possibly by interfering with the activation pathway of IRF-3. A second study revealed that the coding sequence of the S segment of CCHFV contains a proteolytic cleavage site, DEVD, which is conserved in all CCHFV strains. By using different recombinant expression systems and site-directed mutagenesis, it was demonstrated that this motif is subject to caspase cleavage. It was also demonstrated that CCHFV nucleocapsid (N) protein is cleaved into a 30-kDa fragment while caspase activity is induced during infection and that CCHFV infection induces caspase-3-dependent apoptosis at late post infection. Using caspase inhibitors and cells lacking caspase-3, it was shown that the cleavage of N protein is caspase-3-dependent. The inhibition of apoptosis induced progeny viral titres.

A study examining the crystal structures of CCHFV N revealed two distinct forms, an oligomeric form comprised of double antiparallel superhelices and a monomeric form. The head-to-tail interaction of the stalk region of one CCHFV N subunit and the base of the globular body of the adjacent subunit stabilises the helical organisation of the oligomeric form of CCHFV N. It also masks the conserved caspase-3 cleavage site present at the tip of the stalk region from host cell caspase-3 interaction and cleavage. Incubation with primer-length ssRNAs revealed the crystal structure of CCHFV N in its monomeric form, which is similar to a recently published structure. The conformational change in CCHFV N upon deoligomerisation results in exposure of the caspase-3 cleavage site and subjects CCHFV N to caspase-3 cleavage. Mutations of this cleavage site inhibit cleavage by caspase-3 and result in enhanced viral polymerase activity. These structural findings extend current knowledge regarding CCHFV N structure. Based on the high degree of structural similarity between CCHFV and LASV N proteins, they may have an ancestor in common.

A final study showed that CCHFV has strategies for interplaying with apoptosis pathways, thereby regulating the caspase cascade. There were indications that CCHFV suppresses caspase activation at early stages of the CCHFV replication cycle, which perhaps benefits the establishment of infection. Furthermore, the host cellular response at late post infection appears to induce host cellular pro-apoptotic molecules through the death receptor pathway. External host-derived stimuli probably initiate the apoptotic process and the route continues either by crosstalk between the death receptor and mitochondria routes, or separately.

LIST OF SCIENTIFIC PAPERS

I. Andersson I1, Karlberg H, Mousavi-Jazi M, Martínez-Sobrido L, Weber F, Mirazimi A.
Crimean-Congo hemorrhagic fever virus delays activation of the innate immune response
J Med Virol.2008Aug;80(8):1397-404.

II. Karlberg H, Tan YJ, Mirazimi A.
Induction of caspase activation and cleavage of the viral nucleocapsid protein in different cell types during Crimean-Congo hemorrhagic fever virus infection
J Biol Chem. 2011 Feb 4;286(5):3227-34.

III. Wang Y, Dutta S, Karlberg H, Devignot S, Weber F, Hao Q, Tan YJ, Mirazimi A, Kotaka M.
Structure of Crimean-Congo hemorrhagic fever virus nucleoprotein: superhelical homo-oligomers and the role of caspase-3 cleavage
J Virol. 2012 Nov; 86 (22):12294-303.

IV. Karlberg H, Tan YJ, Mirazimi A.
Crimean-Congo hemorrhagic fever replication interplays with regulation mechanisms of apoptosis
In press J Gen Virol. 2014

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INTRODUCTION

ZOONOSES

Zoonotic diseases are caused by various pathogens. Human contact and exposure to both animals and arthropods form the basis of how zoonotic pathogens are transmitted. In some cases pathogens are transmitted by vectors such as rodents, mosquitos, sandflies, midges and ticks, which can carry the organism and thereby allow further transmission to different species. Diseases transmitted by vectors are known as vector-borne diseases. Vectors can be introduced to new geographical areas by: i) animal movements (shipment of livestock or migratory birds) or ii) changes in climate and environment (Woolhouse, Haydon et al. 2005). After mosquitos, ticks are the next most common vector within the arthropods and can transmit a wide range of pathogens (Edlow 2008). Important contributions to increased transmission of tick-borne diseases are often observed when humans accidently enter an endemic region or when climate change in the form of milder winters and warm autumns leads to an increase in mean temperature, allowing ticks to molt faster between different life cycle stages (larvae-nymph-adult). This increases the survival rate of ticks and can potentially enhance circulation of pathogens within the tick population (Gray, Dautel et al. 2009). Environmental changes such as increased area of forest and bush land also contribute, by increasing the habitat available for both ticks and larger hosts. Other important factors are a higher number of wildlife, due to decreased hunting activities, which can lead to a potential increase in transmission of the virus (Papa, Velo et al. 2009). In the absence of any commercial vaccine, prevention of disease relies on good awareness of the links between virus, vector and host and of the potential risk for further viral spread to humans, including human-to-human transmission. It also relies on rapid identification, in order to confirm infection (Figure 1).

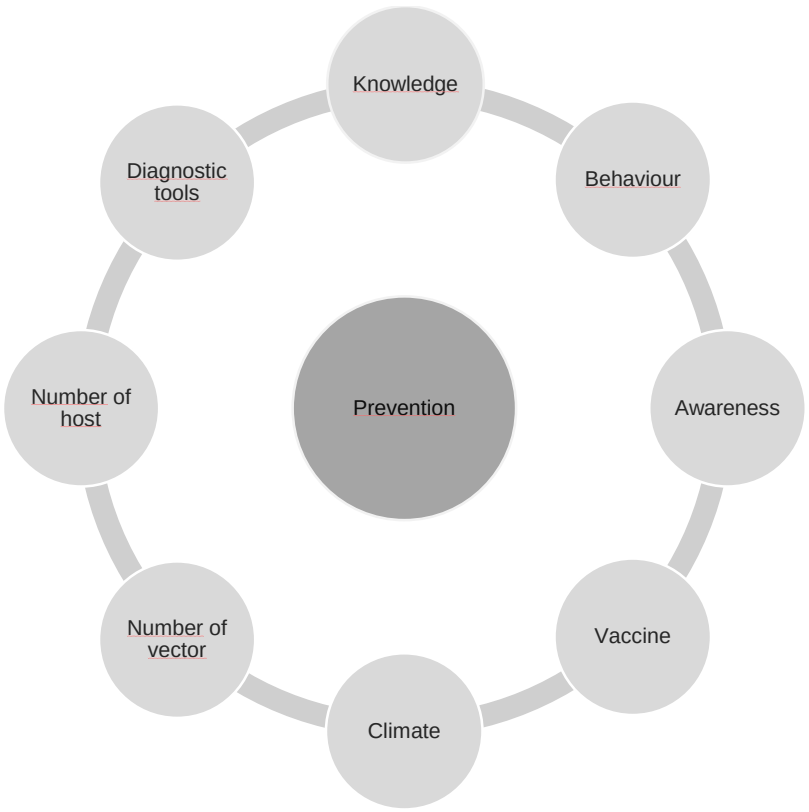

Fig 1. Prevention of CCHF disease relies on good awareness of the links between virus, vector and host and of the potential risk for further viral spread to humans, including human-to-human transmission. It also relies on rapid identification, in order to confirm infection.

Crimean-Congo haemorrhagic fever (CCHF) is a tick-borne viral infection of humans. The CCHF virus, disease or prevalence of antibodies has been reported across a wide geographical area from western China to the Middle East, Balkans and Africa (Ergonul 2006). Humans are the only known host to develop disease after exposure to the virus. The virus can be transmitted by viraemic blood or tissues from patients or by livestock. However, exposure to infectious tick bites seems to be the more common transmission route for humans. There appears to be a connection between the incidence of tick-borne disease, in general terms, and profession. High-risk groups include farmers, veterinarians and forestry workers, i.e. groups regularly exposed to ticks, which results in an elevated risk of tick bites and thereby also disease (Jones, Garman et al. 2002). Nosocomial infections place healthcare workers in danger, especially when sick patients have not received a correct diagnosis.

CCHF disease in humans shows a spectrum of severity, from mild to critical. The pathogenesis of CCHF and other viral haemorrhagic fevers (VHF) has not yet been clearly explained, but appears to be associated with alteration of vascular barrier function, defects in intrinsic coagulation cascade and haemorrhage. These are most probably due to a combination of several factors from the interaction between virus and host cells (Ergonul 2006).

CRIM-CONGO HEMORRHAGIC FEVER VIRUS

VIRUS CLASSIFICATION

CCHFV is a member of the family Bunyaviridae, genus *Nairovirus*. In total, the Bunyaviridae family consists of the genera *Orthobunyavirus, Hantavirus, Phlebovirus, Tospovirus* and *Nairovirus.* CCHFV and all other bunyaviruses except the tospoviruses are zoonotic pathogens transmitted by different arthropods in humans and animals. The genus *Nairovirus* consists of over 30 different viruses, further divided into seven serogroups, but only three members cause disease in humans. These are CCHFV, which is considered to be the most important human pathogen among the nairoviruses, Dugbe virus (DUGBV) and Nairobi sheep disease virus (NSDV) (Elliott 1997, Elliott 2009). Another member of the nairoviruses, Hazara virus, a member of the Crimean-Congo haemorrhagic fever serogroup, but is non-pathogenic for humans. It is classified as risk class 2, whereas CCHFV is risk class 4 but it shares similarities with CCHFV and therefore might be an interesting model for studying CCHFV.

VIRUS STRUCTURE, GENOME ORGANIZATION AND REPLICATION

CCHF viruses are viral particles with a spherical envelope derived from host cellular membrane. The viral genome consists of three single-stranded RNA segments of negative sense referred to as the small (S), medium (M) and large segment (L) (Figure 2)

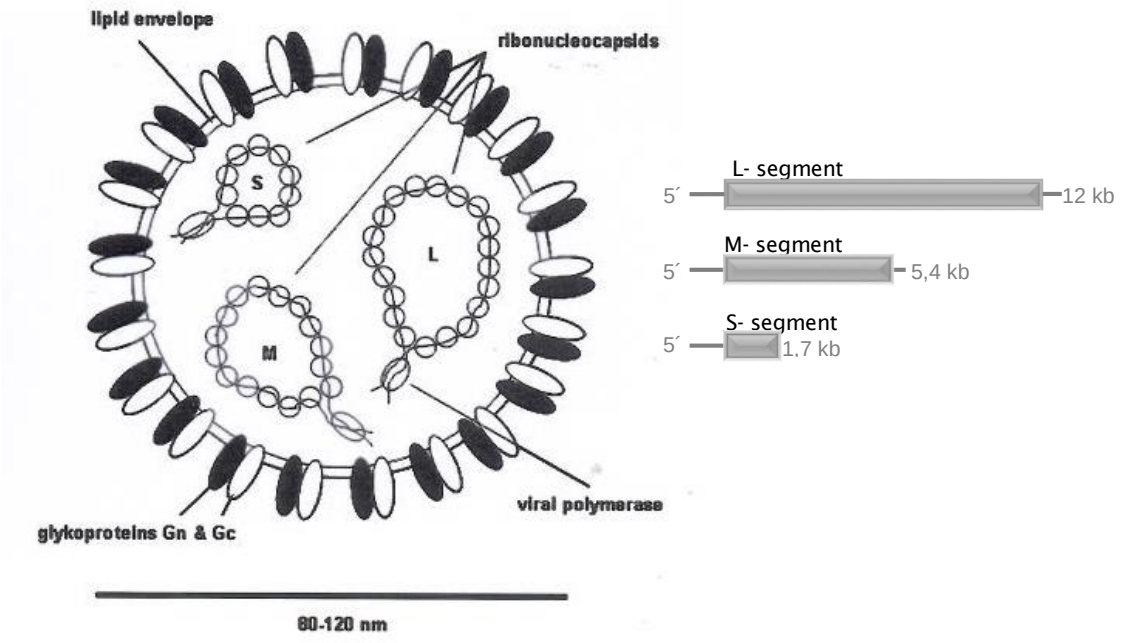

Figure 2. Schematic diagram of the structure of Crimean-Congo haemorrhagic virus (CCHV) (I Andersson)

All three segments contain one open reading frame (ORF) flanked by a non-coding region (NTR/UTR) with partially complementary nucleotids at the end. The S segment encodes for nucleocapsid protein (N) which appears to be multifunctional. Besides protecting the viral RNA by encapsidation, it also participates in viral transcription and replication and is involved in host cellular processes (Elliott 1990, Andersson, Bladh et al. 2004, Andersson, Simon et al. 2004, Simon, Johansson et al. 2009, Karlberg, Tan et al. 2011). The M segment encodes for glycoproteins (Gn, Gc) which protrude through the viral lipid envelope and mediate virus host cell attachment and entry.

RNA-dependent RNA polymerase (RdRp), which is encoded by the L segment, is necessary to initiate transcription and replication. The L segment of CCHFV and other nairoviruses is uniquely large compared with that in other bunyaviruses and contains a ovarian tumour protease domain (OTU), which has been suggested to be involved in cellular anti-viral mechanisms (Frias-Staheli, Giannakopoulos et al. 2007, Bergeron, Albarino et al. 2010). Each RNA segment is individually flanked by terminal noncoding regions (NTR/UTR), conserved between each RNA segment. Within all nairoviruses, these contain partial complementary nucleotides which form circular RNAs referred to panhandle structures. Using a mini-replicon based assay, it has been demonstrated that terminal non-coding regions contain promoter elements necessary for the transcription, replication and encapsidation of each RNA segment (Elliott 1990, Marriott and Nuttall 1992, Flick, Flick et al. 2003).

The receptor for CCHF virus-host cell tropism has not yet been identified, but has been speculated to be a very common receptor or several receptors. Viral glycoproteins mediate the viral attachment step and CCHFV enters through endocytosis (Simon, Johansson et al. 2009). The acidic environment is believed to induce the conformational change in the viral glycoproteins needed for fusion of viral and cellular membranes, which leads to the release of viral ribounucleocapsids (RNPs) into the cytoplasm, followed by viral transcription of the polymerase. Viral RNA serves as a template for both complementary RNA (cRNA) and messenger RNA (mRNA) synthesis and cRNA serves as a template for new viral genomes (vRNA). Shortly after transcription, viral proteins are synthesized on ribosomes, with nucleocapsid protein being the most abundantly synthesized protein in infected cells. Glycoproteins are translated in endoplasmic reticulum (ER), processed and transported to the Golgi complex. nucleocapsid protein, polymerase and newly synthesized vRNA form the RNPs and the assembly and maturation processes of progeny virus occur in the Golgi compartment with glycoproteins incorporated (Sanchez, Vincent et al. 2002). Actin filaments placed within the inner surface of the host cellular membrane play an important role in transporting nucleocapsid protein to the site of assembly, followed by vesicle transport to the cellular membrane where progeny viruses are released into the extracellular space (Elliott 1990, Andersson, Simon et al. 2004).

EPIDIMIOLOGY; OCCURRENCE AND TRANSMISSION

–*Occurrence*

Confirmed CCHF human cases have been reported in south-eastern Europe (Albania, Bulgaria, Greece, Kosovo and Turkey), as well as in the Middle East, Asia and Africa (Maltezou, Andonova et al. 2010, Mertens, Schmidt et al. 2013). Most recently, viral RNA has been found in ticks in Spain (Estrada-Pena, Palomar et al. 2012, Estrada-Pena, Ruiz-Fons et al. 2013).

–*Transmission*

CCHFV is maintained and transmitted by members of the family of Ixodid ticks, particularly the genus *Hyalomma* (Hoogstraal 1979, Shepherd, Swanepoel et al. 1989, Whitehouse 2004). Ticks undergo different stages of their life cycle: beginning from egg, larva, nymph and as an adult (Fig.3). CCHF virus has been isolated from all stages of the tick life cycle, i.e. that transmission may occur both from adult female to egg (vertical) and from larvae to adult (horisontal). Since the life cycle of Ixodid ticks is approximately 2 years, an infected tick can serve as a reservoir for CCHFV by maintaining infection during colder periods and later re-introduce the virus when temperatures rise.

In nature, CCHFV circulates in an enzootic tick-vertebrate-tick cycle in which immature ticks, larvae, need to ingest a blood meal before they can molt to nymphs the next stage of development. Immature ticks feed on smaller animals, while adult ticks also feed on larger animals (Hoogstraal 1979) (Figure 3).

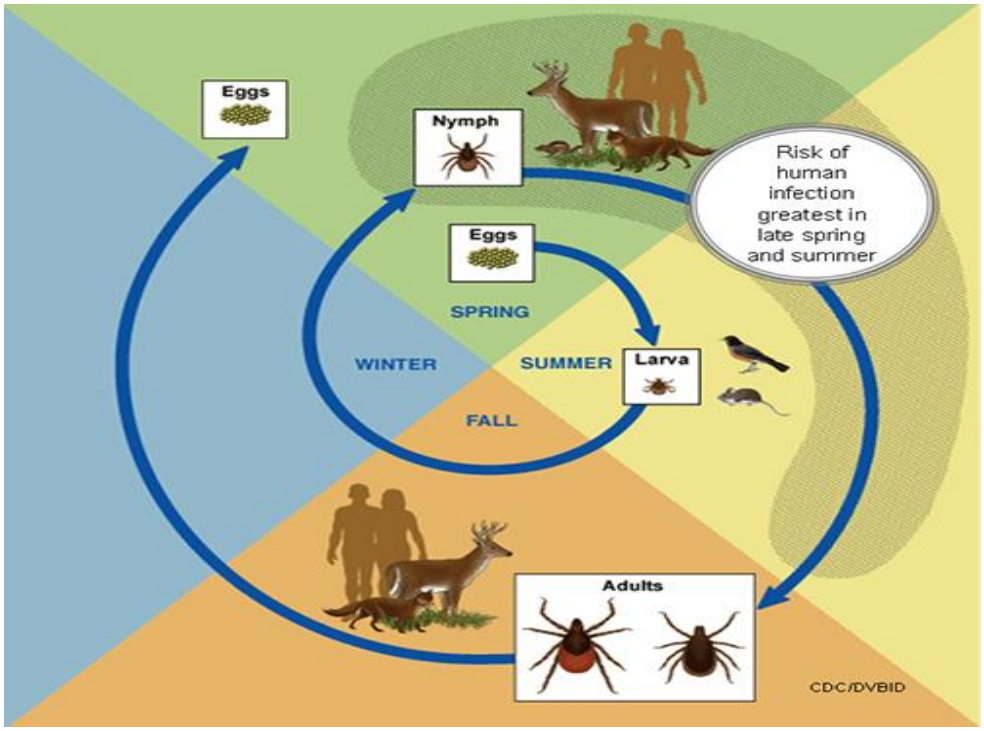

Fig. 3. Life cycle of the Crimean-Congo hemorrhagic fever virus. Hard ticks are both a reservoir and vector for the CCHF virus. The ticks feed on wild and domestic animals (http.//www.cdc.gov)

Ticks spread CCHFV to animals, wild and domestic, which develop a transient viraemia without any signs of illness. In areas where both small (rodents, hares) and large animals (sheep, cattle) are abundant, the virus can circulate unnoticed, with human cases occurring only occasionally. In contrast, in regions that have many small animals but few large animals, any humans who enter the area may be actively hunted by questing, resulting in a large number of infections (Bente, Forrester et al. 2013). This is illustrated by the outbreak of CCHF in Crimea in 1944, during World War II. Due to enemy occupation hunting activities decreased. As a result, the hare population increased enormously, which in turn led to an increase in the tick population. Reoccupation by soldiers led to humans accidentally entering the tick-vertebrate-tick transmission cycle, which resulted in a outbreak of CCHF (Hoogstraal 1979).

Handling livestock and/or contact with blood or tissue from infected animals during the slaughter process increases the risk of transmission of CCHFV. Another risk behaviour which has been identified as a source of infection is tick-crushing play by children in villages in endemic areas (*prof. Z. Vatansever, pers. comm*.). In addition to tick exposure, human-to-human transmission can place e.g. healthcare workers in danger when caring for patients without a proper diagnosis (Shepherd, Swanepoel et al. 1985, Whitehouse 2004, Naderi, Sarvghad et al. 2011) .

DISEASE PROGRESSION

The manifestation of the disease is rapid and can be divided into an incubation, pre-haemorrhagic, haemorrhagic and, among survivors, convalescence phase (Figure 4).

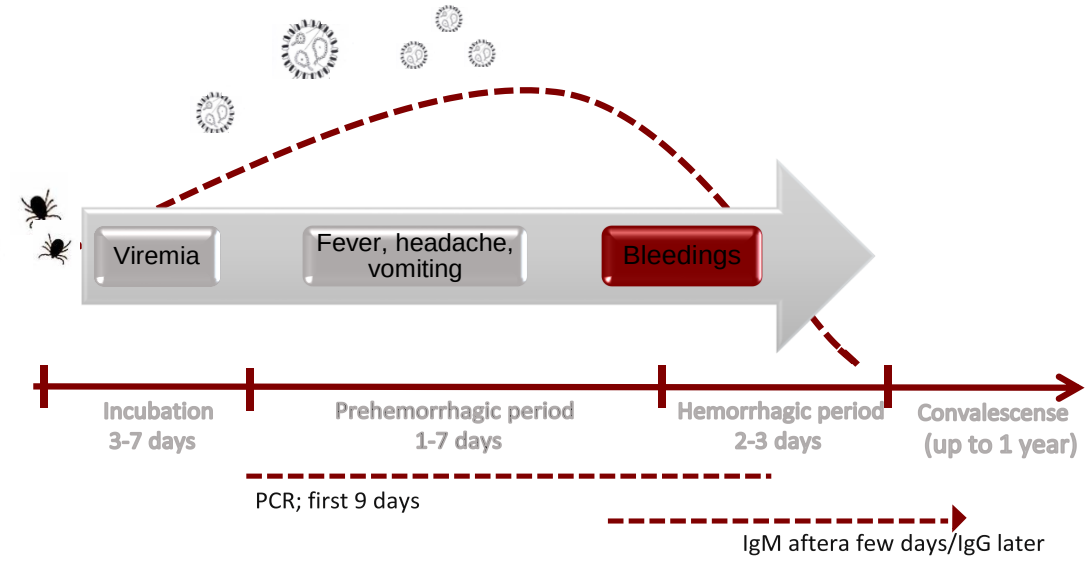

Figure 4. Course of Crimean-Congo haemorrhagic fever (CCHF) infection

The incubation period is usually short and varies between a few days after viral exposure up to one week. There seems to be a correlation between incubation period, viral dose and disease severity. Furthermore, the transmission route appears to be important. Viraemia is suggested to develop faster if the patient has been exposed to a tick bite compared with a nosocomial infection (Swanepoel, Shepherd et al. 1987, Ergonul 2006, Cevik, Erbay et al. 2007). Following incubation, the patient enters the pre-haemorrhagic phase, which is characterized by fever, vomiting, headache, fatigue and weakness. During this period the potential risk of human-to-human transmission is great, placing e.g. relatives, those caring for the sick member of the family or healthcare workers in danger, especially if the patient has not received an accurate diagnosis. The haemorrhagic period is short and is characterized by haemorrhagic manifestations appearing on mucus membranes and on the skin, and in this phase the patient may start to bleed from various sites (Whitehouse 2004, Ergonul 2006). According to the literature, death occurs 6-10 days after the patient first develops clinical signs and is due to organ failure caused by loss of blood, anaemia, dehydration and shock (Swanepoel, Gill et al. 1989). The convalescence period among survivors involves temporary symptoms such as weakness, confusion etc. (Hoogstraal 1979, Schnittler and Feldmann 2003)

The level of viraemia can be used as a prognostic marker. A high level of viral RNA (> 1.10^9 genomes per mL plasma) early during infection is more frequently associated with a fatal outcome (Cevik, Erbay et al. 2007).

Another marker predicting the prognosis of the disease is the development of a humoral response, since fatal cases show little evidence of development of a protective IgG antibody response (Hoogstraal 1979, Ergonul 2006).

PATHOGENESIS OF DISEASE

The mechanism behind the pathogenesis of CCHF is not completely understood, but is associated with alteration of vascular barrier function and haemorrhage, most probably due to a combination of several factors.

Animal studies on Ebola haemorrhagic fever virus, which shares clinical features with CCHF, suggest that release of pro-inflammatory cytokines by the host response contributes to altered vascular function, hypotension, shock and organ failure (Schnittler and Feldmann 2003, Feldmann and Geisbert 2011).

Endothelial cells are considered to be the major target of CCHFV. Endothelial cells line the inner surface of blood vessels and represent a barrier between blood and tissue. *In vivo* studies have provided evidence of CCHFV viral antigen and RNA in endothelial cells from liver and spleen (Bereczky, Lindegren et al. 2010, Zivcec, Safronetz et al. 2013). However, those studies were performed at time of death.

In general, CCHF patients show abnormal function of the coagulation system and leakage of erythrocytes and plasma through the vascular epithelium into the tissue, which contributes to hypotension and in severe cases can lead to shock and organ failure (Burt, Swanepoel et al. 1997). Other symptoms include enlarged liver and spleen, elevated plasma level of liver enzymes, platelet shortage and prolonged bleeding times (Hoogstraal 1979).

Exposure to an infected tick bite seems to be the most common infection route for humans and in this case the first barrier overcome is the epithelium. This is followed by viral entry and release into endothelial cells, which leads on to viral spread in the bloodstream (Jones, Garman et al. 2002, Whitehouse 2004). In animal models, it has been demonstrated that early viral replication occurs in the blood, liver and spleen and thereafter spreads to the lung and kidney and can later also disseminate to the brain (Bente, Alimonti et al. 2010).

The dissemination route of CCHFV has not completely been determined, but it has been suggested that amplification of the virus in macrophages and dendritic cells (antigen presenting cells) might contribute to increased levels of released cytokines, but also facilitate

transmission of the virus to local lymph nodes, the spleen, the liver and the systemic circulation (Martinez, Leung et al. 2012).

Infection leads to degradation or dysfunction of endothelial cells, but it is not known whether this is caused by virus-mediated host factors, by the virus infection itself or a combination of both (Peters and Zaki 2002, Ergonul 2006). Activation of endothelial cells is critical in starting immune reactions and activation of intrinsic coagulation cascade. Altered vascular function helps to enhance movement of circulating leucocytes from the sites of infection, thereby starting an inflammatory process (Weber and Mirazimi 2008).

Infection and activation of DCs, monocytes or macrophages most probably release mediators, which contribute to damage to the endothelium. An overactive host response may be important in the progression of the disease. In severe CCHF cases, high levels of cytokines most probably also contribute to the progression of the disease. *In vitro* studies have demonstrated that macrophages are susceptible to CCHFV and, as a result, increased levels of pro-inflammatory cytokines have been reported (Peyrefitte, Perret et al. 2010). A study of CCHF cases in Turkey found increased serum levels of TNF-α (Ergonul, Tuncbilek et al. 2006). In other studies, high levels of interleukin-6 (IL-6), IL-10 and tumour necrosis factor-α (TNF-α) have been used as prognostic markers in CCHF patients (Papa, Bino et al. 2006, Saksida, Duh et al. 2010)

Haemorrhage is usually observed when thrombocytopenia develops or the function of platelets is suppressed, but the bleeding is usually diffuse and haemorrhage is not alone the major factor responsible for the death of patients. However, viral dose seems to be correlated with a hyperactive host response. When this is combined with impaired immune response such as delayed production of type I interferons (IFNs) and absence of an antibody response, the disease can progress into more severe stages (Saksida, Duh et al. 2010). Endothelial damage and vascular collapse causing coagulation disorders and organ necrosis could be what leads to the shock syndrome and multi-organ failure observed in severe and fatal CCHF cases (Peters and Zaki 2002, Whitehouse 2004, Ergonul 2006).

ANIMAL MODELS

Two animal models with a defective interferon response, IFNAR and STAT1 mice, in which CCHFV infection is lethal, have recently been developed (Bente, Alimonti et al. 2010, Bereczky, Lindegren et al. 2010). These animal models have been used to study the pathogenesis of the disease.The fact that these IFN knock-out mice are prone to fatal CCHFV infection indicates the importance of anti-viral mechanisms in the early innate response. The highest levels of CCHF vRNA are reported to occur mainly in the liver and spleen of IFNAR and STAT1 mice. (Zivcec, Safronetz et al. 2013)These organs are clearly affected, with observations of necrosis in the liver and massive lymphocyte depletion in the spleen, reflecting human disease patterns. Infected mice develop leukopenia, thrombocytopenia and elevated levels of liver enzymes. Furthermore, animal studies of CCHF have demonstrated increased levels of proinflammatory cytokines such as IL-6, IL-10 and TNF-α (Bente, Alimonti et al. 2010, Zivcec, Safronetz et al. 2013)

PREVENTION, CONTROL AND TREATMENT

Prevention and control

In the patient care setting, standard barrier nursing (such as gloves, masks, face shields) and patient isolation are sufficient to prevent transmission of CCHF (Ftika and Maltezou 2013) (Ftika and Maltezou 2013).

Differential diagnosis is important, since early non-specific symptoms of CCHF can mimic other infections, leading to misdiagnosis, increasing the risk of virus transmission and delaying appropriate treatment (Whitehouse 2004).

There is a promising vaccine candidate (Buttigieg, Dowall et al. 2014) however in the absence of any commercial vaccine, prevention of disease relies on awareness regarding the link between the virus, vector and host and the potential risk of further viral spread to humans, including human-to-human transmission In a recent study examining risk factors for tick exposure, it was found that tick bites can be prevented by simple changes in behaviour, by understanding those at risk and by implementing barriers to prevent tick bites (Jones, Garman et al. 2002). It is important to implement preventive and protective behaviour such as proper clothing, with no exposure of naked skin, while the body and clothing should be inspected for ticks after being outdoors. Attached ticks should be removed without damaging the tick body, followed by cleaning the bite area with alcohol or soap and water (CDC, 2011). People in high risk occupations (slaughterhouse workers, veterinarians, shepherds) should avoid virus-contaminated blood or tissue. For farmers, it is especially important to check for tick exposure

after handling livestock (Whitehouse 2004). Russian milkmaids recognised the possibility of infection with tick-borne diseases and therefore dressed from head to toe in white garments to allow them to check more easily for the presence of ticks (Dr Telford Work).

Treatment

General supportive therapy is often the best approach this includes monitoring patient status, administration of fresh frozen plasma to maintain levels of clotting factor, thrombocytes and blood transfusions following haemorrhage (Ergonul 2006).

Administration of Ribavarin is the only medical treatment available and is an option during early infection. However, there is an ongoing debate regarding its antiviral effect (Tasdelen Fisgin, Ergonul et al. 2009). Ribavarin is an nucleoside analogue which has antiviral properties. The complete mechanism underlying this antiviral effect is not known, but it has been suggested to interfere with vRNA synthesis (Graci and Cameron 2006). The first clinical use of Ribavarin against CCHFV was documented in 1995, when three healthcare workers recovered after (Fisher-Hoch, Khan et al. 1995)

As mentioned above, scientists have concluded that administration of Ribavarin is most effective in early infection, during viraemia. Later on, when the patient enters the haemorrhagic phase and viraemia has declined, a stage which is characterized by the release of pro-inflammatory cytokines, sepsis and bleeding disorders, the approach should perhaps be to use drugs or compounds that address these issues (Ergonul 2008).

Use of antibodies (immunoglobulin) from survivor donors may be of therapeutic importance (Hoogstraal 1979). Immunoglobulin has been used as treatment for CCHF patients in both Bulgaria and Turkey, but there is a lack of efficiency studies (control studies) in this field (Christova, Di Caro et al. 2009, Keshtkar-Jahromi, Kuhn et al. 2011, Kubar, Haciomeroglu et al. 2011) .

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HOST RESPONSE TO VIRAL INFECTION

The host response to viral infections can be divided into different stages. The first line of defence includes physical barriers such as skin, low pH and fever that separates and protects the host from the outside world. If the mechanical barrier fails, e.g. the epithelial lining in humans and other mammals, the host still has a variety of defence mechanisms referred to as the immune system. It includes production of specialist cells and secretion of protective molecules/proteins which prevent microbial pathogens from evading the defences and establishing an infection in the host.

The innate arm of the immune system acts quickly and in a blunt manner and therefore responds non-specifically to a large variety of pathogens. The early immune system is important since it helps to limit further spread and virus replication, thereby preventing and controlling the infection until it is eliminated or until the pathogen-specific adaptive arm of the immune system is established. This adaptive immune response is slower, specific and contributes to an immunological memory, which results in faster recognition and response in the event of a secondary infection with the same pathogen (Chaplin 2010). Important events early during infection include secretion of pro-inflammatory mediators, which are produced by most immune cells with the aim of detecting intruders such as pathogens, and activation of the adaptive immune response, which acts together with the innate arm. Chemokines recruit immune cells by chemotaxis, i.e. movement of the immune cells in response to stimuli. Cytokines such as interleukins (ILs) stimulate activation and recruitment of other cells, while IFNs can act in an autocrine or paracrine manner. The purpose of both is to induce expression of antiviral proteins, which helps to put cells in anti-viral mode, reduces cell permissiveness to virus infection and thereby slows down or inhibits virus multiplication. Besides their role as antiviral messengers, IFNs also buy the organism time to establish an adaptive immune response (Commins, Borish et al. 2010). IFNs have a wide array of biological functions including inhibition of cell proliferation, modulation of the immune response and regulation of apoptosis (Le Bon and Tough 2002).

Another host anti-viral strategy is to commit cell suicide by activation of apoptotic pathways. Apoptosis or necrosis can help limit viral replication and the spread of infectious viral particles, and thereby protects the host from invading pathogens. In order for an invading virus to achieve successful viral replication and the production of progeny virus in order to establish infection, it has to be able to evade the apoptosis defence mechanism or use apoptosis as an mechanism which contributes to viral spread.

While apoptosis is an programmed form of cell death, coordinated caspase activation. Necrosis involves acute cellular injury and is an uncontrolled form of cell death, often

considered to be a toxic process. Necrosis is characterized by cell swelling chromatin condensation and rupture and loss of cellular membrane integrity, which results in release of cellular contents into the surrounding tissue, thereby promoting inflammatory reactions

Apoptosis is characterized by cell death without any leakage of cellular contents to extracellular space causing secondary damage. Characteristic cell changes such as cell shrinkage, chromatin condensation and cytoplasmic condensation are followed by plasma membrane blebbing and the formation of membrane-enclosed particles (containing cellular components) known as apoptotic bodies (Elmore 2007). These are further identified and engulfed (phagocytosed) by neighbouring cells. Through the formation of apoptotic bodies, the virus can disseminate without triggering any immune response, facilitating virus release. Since apoptotic cells do not release their cellular components, apoptosis is therefore considered to be a limited or non-inflammatory form of host cell suicide (Wickman, Julian et al. 2012).

CASPASE- DEPENDENT APOPTOSIS

The term apoptosis was initially used to describe a form of cell death associated with specific cellular morphological changes. Since then, molecular mechanisms, induced by both internal and external stimuli, have been characterized. Apoptosis is a cell-programmed suicide mechanism that results in controlled breakdown of cells into smaller apoptotic bodies. Key effector molecules in the apoptotic cascade include the B-cell lymphoma (Bcl)-2 superfamily and caspases. The Bcl-2 family has both pro-and anti-apoptotic members, which help to regulate apoptosis and prevent or induce mitochondrial changes. In inactive form these molecules are localized in the cytosol or present on the mitochondrial membrane (Zimmermann, Bonzon et al. 2001, Zimmermann and Pinkoski 2001).

The cysteine aspartate-specific proteases (caspases) mediate the execution phase of programmed cell death. Caspases are among the most specific of proteases and require specific aspartic residues (Asp) for recognition and binding. Furthermore, different caspases have different specific recognition motifs which involve recognition of surrounding neighbouring amino acids (Thornberry 1998).

The caspases are categorized into different groups (Table 1):

i) Mediators of inflammation: Caspase-1,-4 and -5

ii) Effectors of apoptosis: Caspase-3,-7 and -2'

iii) Activators of apoptosis: Caspase-6,-8,-9 and -10

Table 1. Composition of the three main groups of caspases: The inflammatory caspases mediate cytokine maturation, while the apoptotic caspases are either effectors of cell death or upstream activators of the cell death process

Groups of caspases	**Sequence**	**Function**
Mediators of inflammation		
Caspase-1	W E H D	Activate pro-inflammatory cytokines
Caspase-4	W or L E H D	
Caspase-5	W or L E H D	
Effectors of apoptosis		Function primarly in apoptosis
Caspase-3	D E V D	Cleavage of cellular structural proteins
Caspase-7	D E V D	
Caspase-2	D E H D	
Activators/Initiators of apoptosis		Function primarly in apoptosis
Caspase-6	V E H D	
Caspase-8	L E T D	
Caspase-9	L E H D	

There are two major pathways for initiating apoptosis: the extrinsic pathway, which responds to external stimuli, and the intrinsic pathway (mitochondria-dependent pathway) causing alteration of the mitochondrial membrane function. Both pathways rely on activation of caspases. Activation of execution caspases is controlled by upstream initiator caspases, which are responsible for the apoptotic signalling cascade that finally ends with cell death.

The extrinsic pathway

Cytokines such as Fas ligand (FasL) and TNF-α bind to death receptors, which initiate the extrinsic pathway, activation of initiator caspase-8 and further downstream caspase activation, or via interconnection with the intrinsic pathway by activation of BH3-interacting domain death agonist (Bid). Bid is a proapototic member of the Bcl-2 superfamily and is cleaved and activated by caspases to form a truncated fragment, which then translocates to the mitochondria (Thornberry 1998, Zimmermann, Bonzon et al. 2001) (Figure 5).

The intrinsic pathway

Mitochondria have several important functions, such as production of cellular energy (ATP), regulation of cellular metabolism and several other functions. The intrinsic or mitochondria pathway is associated with changes in the mitochondrial membrane, leading to release of mitochondria proteins, which serve as inducer molecules for caspase activation. Release of mitochondrial proteins can be caused by intracellular stress and by host pro-apoptotic proteins, induced by the extrinsic pathway, that target the mitochondria, causing loss of mitochondria integrity such as swelling, formation of membrane pores and increased permeability of the mitochondrial membrane. There is most probably cross-talk between the extrinsic and intrinsic pathways, for example by activation of Bid-tBid. After translocation to the mitochondria, tBID interacts with cellular pro-apototic proteins which cause these changes in the mitochondrial membrane. Changes in mitochondrial permeability are regulated by anti- and pro-apoptotic proteins such as the Bcl-2 superfamily. Proteins encoded by this family can either promote apoptosis (Bax, Bak) or inhibit apoptosis (Bcl-2, Bcl-xL), by direct action on the mitochondrial outer membrane, allowing leakage of apoptotic proteins such as cytochrome-c. Once cytochrome-c is released, it binds to apoptotic protease activating factor-1 (Apaf-1), which forms the apoptosome and further activates caspase-9, which in turn results in downstream executor caspase activation (Thornberry 1998, Zimmermann, Bonzon et al. 2001) (Figure 5).

Execution phase

The final step of apoptosis ends at the same point for both the extrinsic and intrinsic pathways, namely the execution phase. Execution caspases include caspase-3, caspase-6 and caspase-7, with caspase-3 considered to be the most important. It is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) (Sakahira, Enari et al. 1998). Execution or downstream caspases further cleave various cellular substrates such as poly (ADP-ribose) polymerase (PARP), which is involved in DNA repair and is an indicator of cells

undergoing apoptosis, since caspase cleavage of PARP leads to inhibition of the PARP function (repairing ssDNA nicks) (Thornberry 1998, Thornberry and Lazebnik 1998).

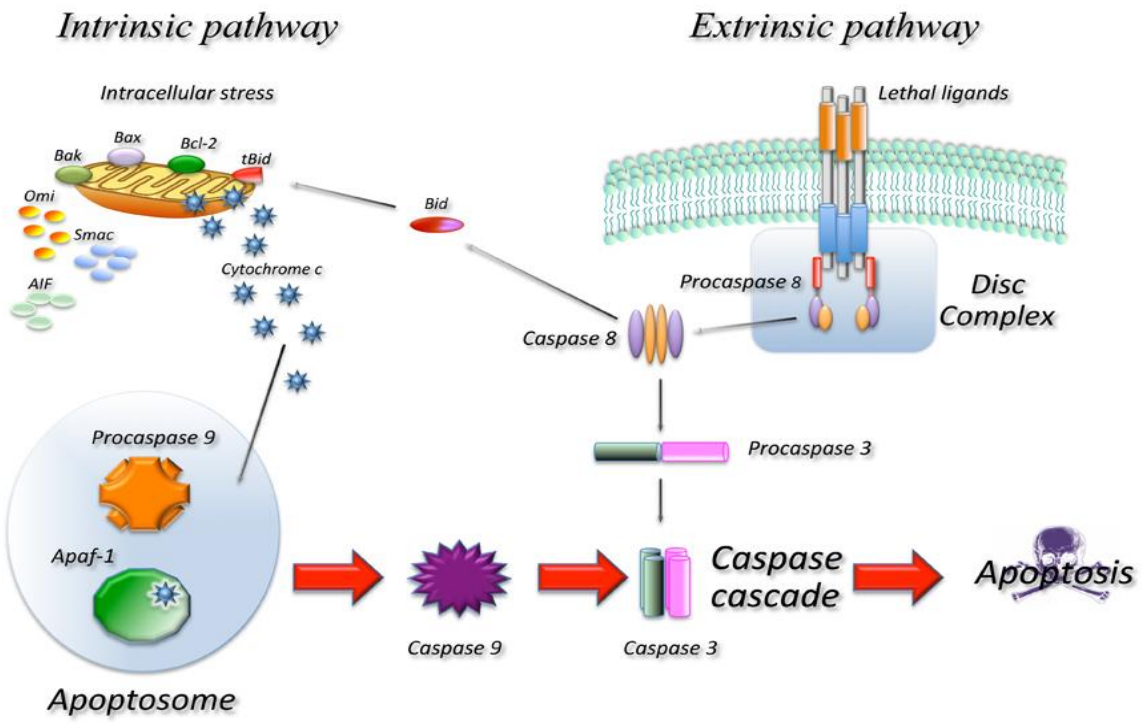

Figure 5. Schematic representation of the main molecular pathways leading to apoptosis (Favaloro, Allocati et al. 2012).

Removal of apoptotic cells

The last step during the apoptotic process is the removal or phagocytosis of apoptotic bodies by neighbouring cells. Phagocytic molecules such as phosphatidyl serine are transferred from the cytosolic inward-facing surface of the plasma membrane to the extracellular surface or outer-facing layer of apoptotic cells. These molecules are markers or recognition signals and allow uptake, “eat me-find me signals”, that result in phagocytosis of dying cells with limited damage to surrounding tissue and thereby minimal immune response (Fadok, de Cathelineau et al. 2001, Wickman, Julian et al. 2012)

Modulation of host antiviral response

Viruses have different strategies to interfere with cell death pathways during infection to secure efficient progeny viral production by expressing viral proteins (encoding gene products) or down-/up-regulating cellular pro- and anti-apoptotic proteins, which interfere with apoptotic signalling pathways.

Apoptosis and/or modulation of apoptopic signalling pathways has been observed for several members of the Bunyaviridae, such as Rift Valley fever virus (RVFV), Bunyamwera virus (BUNV), La Crosse virus (LACV), Oropuche virus (OROV) and Hantaan virus (HTNV) (Won, Ikegami et al. 2007, Kohl, Clayton et al. 2003, Pekosz, Phillips et al. 1996, Colon-Ramos, Irusta et al. 2003, Acrani, Gomes et al. 2010, Gupta, Braun et al. 2013, Ontiveros, Li et al. 2010).

ANTI-VIRAL STATE INDUCED BY INTEFERONS TYPE I

Pattern recognition receptors

Detection of microorganisms in the host is facilitated by a set of intracellular and transmembrane proteins termed pattern recognition receptors (PRR), which recognize structures of pathogens either through pathogen-associated molecular patterns (PAMPs) displayed on the surface or by being a part of their genome. Since many microorganisms express the same PAMPs, the host can detect a large variety of pathogens via PRR. Both extra and intracellular recognition by PRR lead to a signalling cascade, which results in expression and secretion of IFNs and/or cytokines that activate, or mediate the activation of, the adaptive immune response (Takeuchi and Akira 2010).

Extracellular and endosomal recognition

One example of the PRR are the toll-like receptors (TLRs). These transmembrane proteins are either located on the cellular membrane and recognize and bind to microorganism structures such as lipopolysaccharides (LPS) and flagellin, or are located on the endosomes and bind to genetic entities such as the DNA/RNA of micro-organisms. For example, TLR-3 detects double-stranded RNA (dsRNA), TLR-7 and TLR-8 recognize single-stranded RNA (ssRNA) virus genomes and TLR-9 recognizes DNA viruses. TLR-3 is found in the plasma membrane as well intracellularly in the endosomes and TLR-7 and TLR-9 are located in the endosomes of cells (Akira and Takeda 2004).

Intracellular recognition

An example of PRR, that responsible for intracellular recognition of pathogens, is created by the retionic acid-inducible gene-1 (RIG-I) and melanoma differentiation antigen-5 (MDA-5). RIG-I and MDA-5 are the two main intracellular receptors for binding viral RNA upon activation, triggering a signalling chain that activates IFN-β gene expression (Takeuchi and Akira 2007, Yoneyama, Onomoto et al. 2008). Although RIG-I and MDA-5 have a similar protein structure and share a similar signalling pathway, it has been demonstrated these two RNA helicases discriminate in sensing different viral patterns, suggesting a degree of specificity. RIG-I binds to short dsRNA molecules, while MDA-5 recognizes and binds to long dsRNA structures (Kato, Takeuchi et al. 2006, Loo, Fornek et al. 2008). However, this division of mode may be oversimplified, since recent studies have found that RIG-I has the ability to bind to ssRNA with triphosphate at the 5´end of the genome (Hornung, Ellegast et al. 2006, Pichlmair, Schulz et al. 2006)

Inteferon (IFN) induction

Interaction of antigen to PRR starts a signalling chain which results in activation of transcription factors, for example by nuclear factor κB (NF-κB) or IFN regulatory factor-3 (IRF-3). Both NF-κB and IRF-3 are normally held in the cytoplasm in inactive state. For its activation, NF-κB requires degradation of inhibitory proteins, while IRF-3 is activated by phosphorylation of different residues. This allows translocation into the nucleus, where both initiate IFN-β gene expression and secretion of IFN-β (Figure 6). The first wave of IFNs triggers expression of IRF-7, which initiates synthesis of several subtypes of IFN-α. Although there are different types of IFNs, all share the same downstream signalling pathway, the Janus kinase/signal transducers and activators of transcription (JAK-STAT) signalling pathway. Upon binding of IFNs to type I IFN receptor (IFNAR), Janus kinases (JAKs) are activated to phosphorylate, and thereby activate, downstream substrates such as STAT-1 and 2, which together with IRF-9 form a complex called IFN-stimulated gene factor 3 (ISGF-3). It in turn induces transcription of several IFN-stimulated genes and a second wave of IFN-α/β expression (Smith, Lombardi et al. 2005, Weber and Mirazimi 2008).

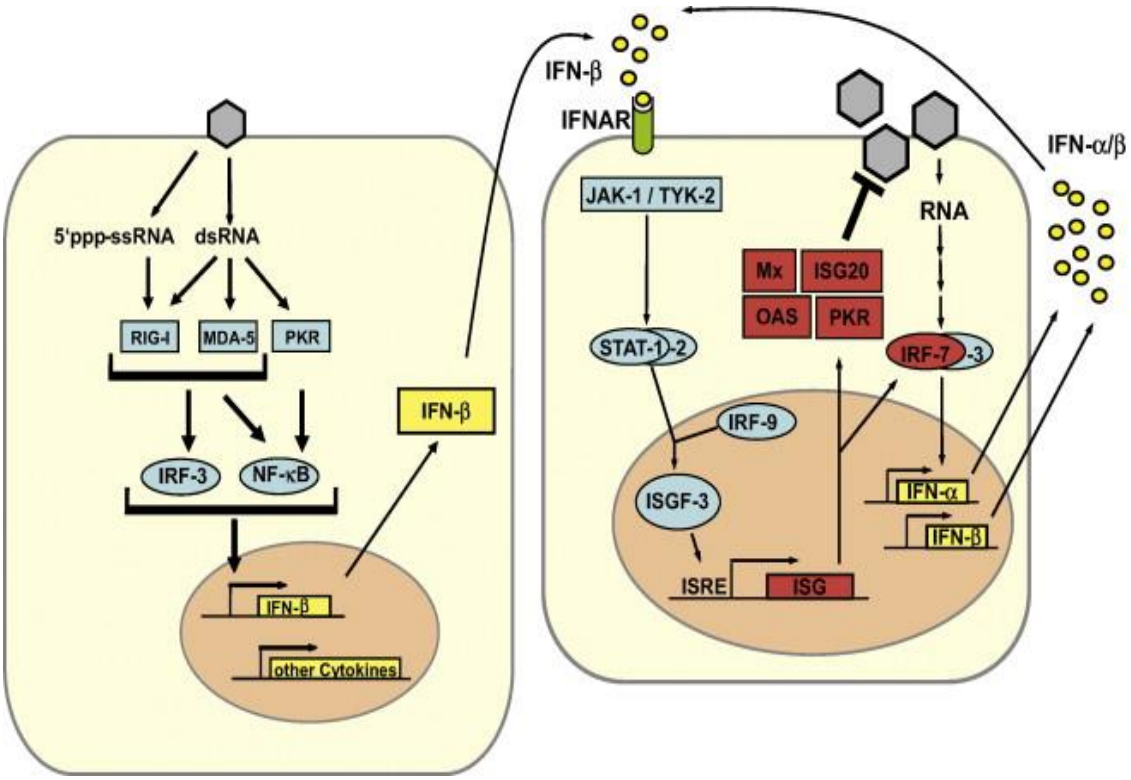

Figure 6. Type I interferon (IFN) induction, signalling and release (Weber and Mirazimi 2008).

Inteferon stimulated genes (ISG) products

Synthesis of IFNs is considered to be efficient and rapid host response and is important for clearance of virus infection. Secreted IFNs can stimulate neighbouring cells to express antiviral proteins. IFNs can contribute to induction of an antiviral state in host cells and thereby help the host to limit viral replication and further viral spread (Haller, Kochs et al. 2006). A few proteins have been characterized and are reported to be induced by a set of genes with antiviral effect, referred to as interferon-stimulated genes (ISGs) (Balachandran, Kim et al. 1998).

One of the ISGs up-regulated following IFN stimulation is IRF-7, which in turn initiates IFN-α genes and thereby production of a full range of IFNs. Other IFN-induced antiviral proteins are protein kinase R (PKR), Mx GTPases such as human MxA and ISG15-ubiquitin-like molecules, the 2´-5´oligoadenylate synthetases (OAS) and ISG20.

The Mx proteins are present in a number of species, including humans. The human MxA interferes with viral replication soon after entry to the host cell upon sensing dsRNA, and can interact with viral nucleocapsid proteins of members of the Bunyaviridae. In this manner they restrict their cellular location, preventing nucleocapsids from taking part in (their normal function of) viral RNA synthesis and thereby limiting viral replication (Zurcher, Pavlovic et al. 1992, Kochs, Janzen et al. 2002, Andersson, Bladh et al. 2004, Haller and Kochs 2011).

PKR is activated by viral dsRNA and 5´triphosphate group ssRNA and blocks viral mRNA translation and general host protein synthesis by phosphorylation of the eukaryotic initiation factor 2α (eIF2α) by PKR (Zhu, Romano et al. 1997, Sadler and Williams 2008).

Cellular exonucleases are important for the protection against pathogens. Activation of RNaseL contributes to degradation of viral RNA and thereby prevents virus replication and protein synthesis. Another cellular exonuclease, ISG20, seems to be a part of this antiviral effect by degrading ssRNA. Besides the direct antiviral effects, PKR also mediates apoptosis and regulates cell proliferation (Gil and Esteban 2000)

Viruses are intracellular organisms, meaning that they can only reproduce within a host and need to use host cellular replication machinery for multiplication and to ensure transmission and spread to new hosts. For this to be achieved in the presence of early, innate, antiviral defence mechanisms, most viruses have evolved strategies to interfere with the IFN response. The presence of virus antagonists designed to interfere with different signalling pathways, such as the IFN response, enables viruses to replicate and secure progeny virus production and thus continue the course of infection.

Viral encoded IFN antagonists can target specific components of the IFN system by suppressing, delaying or inhibiting induction of IFN signalling or inhibiting the effects of antiviral

protein induced by ISGs. In addition, some viruses block or shut-off general protein synthesis in order to ensure their survival.

One role of non-structural **(**NSs) proteins for both phleboviruses and orthobunyaviruses is to act as inhibitors of the host antiviral response. Rift Valley fever virus NSs target the antiviral response by blocking IFN production early during the viral replication cycle by inhibiting IFN-β gene transcription and interfering with or down-regulating the antiviral protein PKR (Bouloy, Janzen et al. 2001, Billecocq, Spiegel et al. 2004).

The NSs of both BUNV and LACV block the IFN response on host transcriptional level by targeting RNA polymerase II (Thomas, Blakqori et al. 2004, Verbruggen, Ruf et al. 2011)

Another strategy to counteract the host early immune response and IFN signalling pathways is to avoid intracellular RIG-I recognition, a survival tactic demonstrated for members of the hantaviruses and nairoviruses. The major determinant of RIG-I activation is 5´triphosphate, a structure that is not normally present in cellular proteins in viral RNA and that is not activated by the CCHFV genome, which instead generates 5´monophosphate (Habjan, Andersson et al. 2008, Weber, Gawanbacht et al. 2013).

Viruses use different strategies to interfere with the IRF-3 pathway. The Ebola virus and hepatitis C virus inhibit IRF-3 phosphorylation (Basler, Mikulasova et al. 2003) (Foy, Li et al. 2003) CCHFV also inhibits IRF-3 activation early during the replication cycle, but further research is needed to characterize the mechanism and determine whether this delay reflects the avoidance of RIG-I recognition early during the virus replication cycle (Andersson, Karlberg et al. 2008).

AIMS

To investigate

- Whether CCHFV interferes with the early host immune response during infection (Paper I)

- The role of conserved DEVD motif and investigate the outcome of caspase activation and cleavage of CCHFV nucelocapsid protein in different human cell types during infection (Paper II)

- The possible role of caspase-3 cleavage sites by using a structure of CCHFV nucelocapsid protein (Paper III)

- How the CCHFV replication cycle interacts with apoptotic pathways (Paper IV)

RESULTS

Paper I

CCHFV delays induction of Type I interferon in infected cells

To address whether replication of CCHFV interferes with the IFN response, we established a bioassay based on a recombinant, IFN-sensitive, New Castle Disease virus (NDV) expressing green fluorescent protein (GFP). In this bioassay, no biologically active type I IFN was found in supernatant harvested 24 hours post infection. However, at 48 hours post infection, substantial amounts of IFN were found in the media (Fig. 1). RT-PCR analysis of the infected cells supported the findings from the bioassay (Fig.1).

In order to investigate whether CCHFV had the ability to interfere with IFN responses, we compared the expression of ISG56 following CCHFV infection to UV-irradiated virus infection. ISG56 is a highly sensitive IFN-stimulated gene and a useful marker for early immune responses. A549 and Huh-7 cells were infected with native and UV-treated virus and analysed with RT-PCR at different times post infection. In both cell types, ISG56 mRNA was detected at 3 hours post infection in cells infected with UV-treated virus. However, in cells infected with native CCHFV, ISG56 mRNA was not detected until 24 hours post infection (Fig. 2)

CCHFV delays activation of IRF3

In addition, we investigated the ability of CCHFV to interfere with IRF-3 nuclear translocation. In an immunofluorescence analysis, we found that IRF-3 was translocated to the nucleus after only 3 hours in cells infected with UV-irradiated CCHFV. In contrast, in cells infected with CCHFV, nuclear translocation of IRF-3 was not observed until 24 hours post infection (Fig. 3)

Paper II

CCHFV N has a specific cleavage motif for caspase-3

Examination of the primary structure of CCHFV N demonstrated a potential motif for caspase cleavage (DEVD) at position 266-269 of the nucleocapsid (N) protein. Comparison with available sequences of S segment of CCHFV in the gene bank revealed that this motif was conserved in all available strains (Fig. 1). To determine whether this motif in N is cleaved during apoptosis, recombinant SFV was produced in order to express CCHFV N. BHK cells were infected with recombinant SFV (moi=1) and cells were harvested at different times post-infection. As shown in Fig. 2, in contrast to mock-infected cells, the cleavage form of PARP was present at 24 hours and was more pronounced in cells collected at 48 hours post infection, which indicates activation of caspase-3. In addition to the expected full-length protein of 56

kDa, several fragments of NP were detected, including a smaller 30 kDa fragment of N at 48 hours post infection (Fig. 2).

In order to confirm that the DEVD motif (Fig. 1) is a subject for caspase cleavage, we created two mutants of CCHFV N, lacking the predicted cleavage site of N. BHK cells were infected with rSFV-expressing mutants and the native form of N, cells were harvested and the expression pattern of N was analysed (Fig. 2).

Caspase activation at late times post infection

To determine whether the cleavage of N occurs during CCHFV infection, SW13, HUVEC and A549 cells were infected with CCHFV (moi=1). Supernatant was collected at different times post infection and analysed. As shown in Fig. 3, we were able to demonstrate the appearance of a cleaved 30 kDa N product in the culture supernatant at 48 hours post infection. However, this fragment was more strongly manifested at 72 hours post infection.

To determine whether CCHFV infection induces apoptosis, we performed a TUNEL assay (DNA strand breaks). SW13 cells were mock-infected or infected with CCHFV (moi=1). At 72 hours post infection, cells were collected and analysed by TUNEL assay (Fig. 4). The results demonstrated that CCHFV infection induced significantly more TUNEL-positive cells compared with mock-infected cells.

To study cleavage of N in more detail, SW13 cells were mock-infected or infected by CCHFV (moi=1). Cells were harvested and analysed for detection of PARP and caspase-3. As shown in Fig. 5, we observed cleavage of PARP and N at 72 hours post-infection. We were also able to demonstrate the presence of activated caspase-3 in infected cells at the same time (Fig. 5).

In order to confirm that the cleavage process of N is caspase-3-dependent, SW13 cells were mock-infected or infected with CCHFV (moi=1) and after 1 hour cells were mock-treated or treated with caspase-3 inhibitors at different concentrations. Cells were harvested at 48 and 72 hours post infection. As shown in Fig. 6, we were able to clearly demonstrate that caspase-3 inhibitor completely inhibited cleavage of N. To confirm these results, we infected MCF-7 cells, which do not express caspase-3, with CCHFV (moi=1). Cells were harvested at different times post infection and analysed. We did not find the 30 KDa fragment of N, but we were able to detect cleavage of PARP (Fig. 6).

To study whether caspase-3 induced apoptosis has an inhibitory effect on yield of progeny virus, SW13 cells were infected with CCHFV (moi=1). These infected cells were then mock-treated or treated with caspase Inhibitors. Supernatant was collected at 24 and 48 hours post infection and the amount of infectious virus particles determined (Fig. 7).

Paper III

CCHFV RNA segments are encapsidated by the nucleocapsid protein (CCHFV N) to form the ribonucleoprotein complex

In analyses of the crystal structures of the CCHFV N, we found two distinct forms, an oligomeric form comprised of double antiparallel super helices and a monomeric form. We also demonstrated that the conserved caspase-3 cleavage site is located at the top, α12-α13 loop, of the stalk domain. This domain is directly involved in the head-to-tail interaction of the CCHFV N molecules and leads to the formation of the superhelical oligomeric form of CCHFV N (Fig. 2). Modelling studies on the interaction with caspase-3 at the caspase cleavage site of CCHFV N in this superhelical form revealed that the head-to-tail interaction of CCHFV N molecules obscures the caspase-3 cleavage site and thus would prevent binding and subsequent cleavage by caspase-3 (Fig. 2).

Incubation with primer-length ssRNA results in conformational change of oligomeric CCHFV N and release of monomeric CCHFV N

When CCHFV N was crystallised in the presence of primer-length ssRNAs (12-16 base long ssRNAs), the structure crystallised under different conditions and only one CCHFV N molecule was observed in the asymmetrical unit (Table 1).

Superposition of the monomeric CCHFV N structure on the molecules from the superhelical structure revealed that a conformational change had indeed occurred upon incubation with primer-length ssRNAs. Rotation of the stalk domain led to destruction of the superhelical oligomer and, as a result, release of monomeric N. This release of the free, monomeric N is likely to result in the exposure of the caspase-3 cleavage site situated at the loop region at the tip of the stalk domain to host cell caspase-3. To confirm this hypothesis, we incubated purified recombinant N with or without ssRNA followed by treatment with caspase-3. Cleavage of CCHFV N by caspase-3 was observed only in the presence of primer-length ssRNA (Fig. 4).

Mutation of the CCHFV N caspase-3 cleavage site enhances viral RNA synthesis

In order to determine the effect of the caspase-3 cleavage site of CCHFV N on viral RNA synthesis activity, we performed minireplicon assays using wild-type CCHFV N or a CCHFV N mutant with the conserved cleavage site mutated to evade caspase-3 cleavage. The results demonstrated that on abolishing the caspase-3 cleavage site of CCHFV N, RNA synthesis by CCHFV polymerase was enhanced (Fig. 5). We also attempted to detect cleaved wild-type CCHFV N fragments by Western blotting, but no cleavage products were detected,

presumably because concentrations of cleaved N from the minireplicon assay were below detectable levels.

Paper IV

CCHFV replication suppresses caspase activation both upstream and on executor caspase level

Mock-infected and CCHFV-infected SW13 cells (moi 1 at 24 h post infection) were treated with different concentrations of STS, a relatively non-selective protein kinase inhibitor, which is often used as a general method for inducing apoptosis. At 5 hours post treatment, cells were harvested and analysed by Western blot. It was found that cleavage of PARP was clearly suppressed in the CCHFV-infected cells compared with the mock-infected cells, in the 4 uM STS treatment (Fig. 1).

To investigate whether caspase-3 activation is regulated during CCHFV infection, SW13 cells were infected and treated as described above (with 4uM STS) and analysed for the presence of caspase-3 cleavage. It was found that the activation of caspase-3 in the STS treated CCHFV infected cells was significantly reduced compared with STS treated mock-infected cells (Fig. 2).

To further characterise the interaction of CCHFV replication and the apoptosis pathway, we analysed these samples for STS-induced cleavage of caspase-9. The results showed that cleavage of caspase-9 was also suppressed during the early phase of the CCHFV replication cycle in STS-treated infected cells, in contrast to mock-infected cells (Fig. 2).

CCHFV infection interplays or interferes with apoptotic molecules-pathways

Mock-infected and CCHFV-infected cells (at 24 h post infection) were mock-treated or treated with STS. Attached and detached cells were harvested at 5 h post treatment, and the cytosol and mitochondria fractions were collected separately and analysed for estimated release of cytochrome-c by Western blot. It was found that release of cytochrome-c into the cytosol was decreased in infected cells compared with mock-infected cells, for both attached and detached cells (Fig.3).

To investigate whether the CCHFV N protein is involved or contributes to regulatory properties, we established an *in vitro* model system The SW13 cells were transfected, with a plasmid coding for ORF N (myc-N), different concentrations, and a plasmid coding for Bax (flag-Bax). Induction of apoptosis in the presence and absence of CCHFV N was then analysed by measuring the activation of caspase-3. Overexpression of Bax in SW13 cells induced high level of caspase-3 activation and cleavage of endogenous PARP, when compared with mock transfected cells. Caspase-3 activity decreased with increasing concentration of N (Fig. 4).

Potential mechanisms contributing to induction of apoptotic pathways

SW13 were mock-infected or CCHFV-infected (moi 1). At 24 and 48 h post infection, cells were harvested and analysed for activation and cleavage of Bid (truncated Bid, tBid). The results clearly demonstrated that CCHFV infection at late post infection led to cleavage of Bid to tBid in cells (Fig. 5).

Supernatant from mock-infected and CCHFV-infected cells at late times post infection was UV-inactivated and transferred to seeded new SW13 cells. At 24 h, cells were harvested and analysed by Western blot for presence of PARP. The results showed that activation and cleavage of PARP was induced in cells that had been treated with supernatant from cells at late post infection (Fig. 6). This is most likely caused by pro-inflammatory mediators released into the medium as a cellular protective response to infection. This suggests in turn that caspase activation is triggered by external stimuli as a secondary effect. Analysis of supernatants of SW13 cells harvested at different times post infection demonstrated that CCHFV-infected cells secreted TNF-α at late post infection.

In order to characterise the process in more detail, supernatant collected at 48 h post infection from infected cells was mock-treated or treated with neutralising TNF-α antibodies (5 ug/mL) for 1 h at 37^{o}C and transferred to seeded SW13. At 24 h post treatment, detached cells were harvested and analysed with Western blot. The analysis showed that antibodies against TNF-α suppressed cleavage of PARP (Fig. 6), indicating that TNF-α secreted from infected cells is a contributor to induction of the extrinsic apoptotic pathway during the late phase of infection. However, the neutralisation was not complete, most probably because we could not neutralise all TNF-α or because other molecules are also involved in triggering the extrinsic pathway.

DISCUSSION

CCHFV N has a conserved caspase-3 cleavage site

CCHF disease in humans shows a spectrum of severity, from mild to acute. The specific underlying mechanism of both the clinical and molecular pathogenesis is still unknown for CCHF and other VHF. Knowledge regarding CCHFV-host cell interaction is also limited therefore the work presented in this thesis focused on understanding how CCHFV replication in host cells interplays with apoptosis and uncovering the mechanism behind this process. **Paper II** demonstrated a potential motif for caspase-3 cleavage (DEVD) at positions 266–269 of the nucleocapsid protein. Comparison with available sequences of the S segment of CCHFV in GenBank™ revealed that this motif was conserved in all available strains. By studying the crystal structures, **paper III**, of CCHFV N, we observed two different conformations of N namely, a double superhelical oligomeric form and a monomeric form. Monomeric N protein is presented as a large globular domain with a protruding stalk domain, where the DEVD site is found in the middle section of N which forms the extended arm. In this state the caspase-3 cleavage site is placed on the most accessible position on the entire molecule and not protected for caspase cleavage. The globular domain has been demonstrated to be important for viral RNA binding. In contrast to the monomeric form, the superhelical oligomer of N the DEVD site is obscured and not available for binding to caspase-3. N protein free as a monomer might be one way for the polymerase to get access to vRNA for transcription and replication.

We, furthermore, demonstrated that the polymeric N undergo a conformational change upon incubation with primer-length ssRNAs (**Paper III**). The major change involved in this process was rotation of the stalk domain, with head-to-tail interaction allowing release of free monomeric N. We hypothesised that release of this free monomeric N would result in exposure of the caspase-3 cleavage site situated at the tip of the stalk domain to host cell caspase-3. To confirm this hypothesis, we incubated purified recombinant N with or without ssRNA followed by treatment with caspase-3. Cleavage of CCHFV N was observed only in the presence of primer-length ssRNA. This confirms our observations in the crystal structure studies, whereby the presence of primer-length ssRNA would cause exposure of the caspase-3 cleavage site of CCHFV N by inducing a conformational change in the protein.

It is interesting to speculate how the interaction between N and these short RNA (primer-length) mimics a step of the CCHFV life cycle in mammalian cells. One might suggest that interaction of primers to RNP, may induce the early step of replication of CCHFV in cytosol. Bunyavirus transcription are suggested to rely on primers generated from capped host-cell mRNAs (Carter, Surtees et al. 2012). Therefore, it is also possible that primer-length RNA

represents mRNA caps to initiate transcription-mRNA synthesis. Our results suggest the interaction between N and RNA regulates the conformation of N and determines if the DEVD motif is exposed for caspase cleavage.

By developing several *in vitro* model system based on different human cell lines, we found that N underwent cleavage, forming two cleavage products in the late CCHFV replication cycle. Using caspase-3 inhibitor and MCF-7 cells, deficient in caspase-3, we found that cleavage of N to a 30-kDa fragment is due to caspase-3 cleavage. Furthermore, we found that caspase inhibitors increased the yield of progeny virus. These findings suggest that caspase cleavage of N does not favour the production of new infectious viral particles. Therefore, we suggest that this event is induced by the host cell as a protective response to CCHFV infection. Viruses have different strategies to interfere or interplay with the host immune response. For example, transmissible gastroenteritis coronavirus (TGEV) infection results in caspase activation and the TGEV nucleoprotein (N) undergoes cleavage in parallel with induced apoptosis. It has been demonstrated that the N protein is a substrate for both caspase-6 and -7 and it is suggested that this could be a general mechanism by which infected cells eliminate coronaviruses, since only the full-length N protein is incorporated into the coronavirus particles. However, production of virions might depend on the ability of the virus to replicate rapidly, since the cleavage process appeared to have a limited influence on viral yields (Eleouet, Slee et al. 2000).

On the other hand, the cleavage of N by caspases appears to be important for the life cycle of some viruses. Influenza A N protein also has caspase cleavage sites, which have been shown to be important for the viral life cycle. Studies using a reverse genetic system have demonstrated that mutations of the N-and C-terminal cleavage motif never lead to rescue of recombinant virus, which suggests that this alteration leads to no surviving viral particles. (Zhirnov, Konakova et al. 1999, Zhirnov and Syrtzev 2009).

To further study the role of the DEVD site on N, we generated CCHFV N with a mutation at the DEVD site. By using a minireplicon assay (**Paper III),** we demonstrated that RNA synthesis activity of the polymerase is higher in DEVD-mutated N compared with wild-type N. This is most probably, due to that the mutated form of N, cannot undergo degradation and thereby there are more full-length protein available for replication process. The results of the minireplicon assay were consistent with our previous findings that titres of CCHFV decreased, when N was targeted by caspase-3 cleavage (**Paper II**). Hence, it is possible that cleavage of N is a way for the host response to slow down virus multiplication and thereby gain time to mount an effective anti-viral response, or it may just accidently happen and have no real purpose. Alternatively, it is possible that the concentration of full length N is important for transition from transcription to replication which further suggests that it is a

way for the virus to down regulate its own expression in order to evade host response (Flusin, Vigne et al. 2011).

Since our results suggest that the DEVD motif is not beneficial for CCHFV replication, one may expect that a fast mutating RNA virus would eliminate the motif easily if there is no fitness cost. However, as the DEVD is conserved across different strains of CCHFV, we hypothesize that the cleavage of N or the DEVD motif itself may play a role in other aspects of CCHFV infection. Firstly, the cleavage products may play a role in the pathogenesis of the CCHF disease. Secondly, our crystallography studies show that the DEVD motif is directly involved in the head-to-tail interaction of N in the oligomeric form and thus it may be involved in controlling the dynamics of conformation changes between the oligomeric and monomer forms of N. This dynamic is critical because the oligomeric and monomeric forms have different importance at different stages of the virus life-cycle.

Last but not least, we should highlight that CCHFV is a tick borne virus, thereby it is most possible to believe that there are other functions related to this cleavage site in replication cycle in tick cells. Humans are dead end host (accidently become infected) and replication in mammals is transient, while ticks remain infected throughout their several-year lifetimes, why it is reasonable that ticks has developed strategies to control infection and perhaps CCHFV during evolution has also developed strategies to suppress or even take advantage of host-tick defense and can live with the ticks in symbiosis.

CCHFV delays/inhibits intrinsic apoptotic pathways

By performing several different analyses on infected SW13 cells, we demonstrated that caspase-dependent apoptotic pathways are induced at late CCHFV infection (**Paper II**). However, since caspase activation does not favour CCHFV progeny viral production, we examined what happens early in the viral replication cycle. We found that apoptotic signalling pathways are regulated by CCHFV early during CCHFV infection (**Paper IV**). By challenging CCHFV-infected cells at 24 h post infection with STS, which is known to induce apoptosis, we were able to show that activation both upstream and at executor caspase level (caspase-9 and caspase-3) was suppressed by CCHFV replication.

To determine what causes the regulation or suppression of apoptotic pathways, we investigated whether changes in mitochondria membrane integrity could be involved in triggering apoptosis by release of pro-apoptotic proteins such as cytochrome-c from the inter-membrane space into the cytosol (Zimmermann, Bonzon et al. 2001). The cytochrome-c and Apaf-1 released form a complex known as the apoptosome, leading to caspase-9 activation,

which further activates downstream effector caspases (Zimmermann, Bonzon et al. 2001). In **Paper IV**, we demonstrated that secretion of cytochrome-c from the mitochondria into the cytosol was decreased in STS-treated CCHFV-infected cells, but not in control cells. This indicates that the release of mitochondria proteins is regulated and caspase activation downstream of mitochondria level, including caspase-3, is suppressed or delayed during the early phase of CCHFV infection.

It has previously been demonstrated that hepatitis C virus (HCV) encodes proteins with anti-apoptotic activity which interfere with apoptosis signalling pathways by different mechanisms. HCV NS2 interferes with caspase-dependent induced apoptosis by counteracting cytochrome-c release from mitochondria, which contributes to viral persistence by interfering with host cell defence (Erdtmann, Franck et al. 2003). In the mitochondrial pathway, pro-apoptotic members of the BCL-2 super family are associated with the mitochondria and release of cytochrome-c. Bax is one of the key pro-apoptotic (BCL-2 member) molecules, an inactive monomer, and is normally found in the cytosol or loosely bound to the mitochondria membrane. Upon activation by apoptotic stimuli, Bax changes confirmation and forms oligomers, which are then integrated by formation of membrane pores that facilitate release of mitochondrial proteins such as cytochrome-c, which leads to downstream caspase activation (Wolter, Hsu et al. 1997, Antonsson, Montessuit et al. 2000).

By using a recombinant expression system, we showed that the over-expression of CCHFV N could inhibit Bax-induced apoptosis (**Paper IV**). These results indicate that the CCHFV N has the ability to protect against apoptosis by acting at the level of Bax or downstream of it. Hence N could be one of the major factors controlling caspase pathways and cell death in the early phase of infection.

It has recently been demonstrated that rubella virus (RV) capsid protein interferes with import of Bax into mitochondria and thereby prevents mitochondria membrane permeabilisation and promotes cell survival (Ilkow, Weckbecker et al. 2010). It has also been reported that Hantaan virus (HTNV) nucleocapsid protein modulates apoptosis pathways through NF-ƙp (Ontiveros, Li et al. 2010).

As discussed above, this thesis provides evidence that at early post infection, CCHFV infection is able to inhibit caspase-3 activation through regulation of cytochrome-c release by interplaying or interfering with Bax. External stimuli through the death receptor pathway might initially induce caspase activation, triggered by the viral infection, and often result in crosstalk between the extrinsic and intrinsic cell death pathways through the pro-apoptotic member Bid, thereby facilitating induction of the mitochondrial pathway (Eleouet, Chilmonczyk et al. 1998, Perez and White 2000). Transmissible gastroenteritis virus infection induces both the Fas L and mitochondria pathways through interconnection with Bid (Ding, Xu et al. 2012).

Role of innate immune response and inflammatory factors in CCHFV-induced apoptosis

In Paper IV, we demonstrated that Bid is active at late post infection and also that supernatant of CCHFV-infected cells contains pro-inflammatory factors, which can induce extrinsic apoptosis. Taken together, these findings indicate that CCHFV at late post infection most probably induces apoptosis through induction of pro-inflammatory factors, which in turn activates the extrinsic apoptotic pathway. This assumption is in line with the finding that UV-inactivated supernatant collected from CCHFV-infected cells at late post infection can induce cleavage of PARP in non-infected cells as early as 24 h post treatment. Since all the supernatants collected were UV-inactivated before being transferred to the fresh cells, apoptosis could not have been induced by virus replication. Induction of apoptotic pathways is most likely caused by pro-inflammatory mediators released into the medium as a cellular protective response to infection. This suggests in turn that caspase activation is triggered by external stimuli as a secondary effect after virus replication.

This thesis also demonstrated that neutralising TNF-α antibodies reduce the cleavage of PARP triggered by supernatant from infected SW13 cells at 48 h post infection. However this needs to be investigated further, since the neutralisation was not complete, most probably because we could not neutralise all TNF-α and/or because other molecules are involved in triggering the extrinsic pathway. Previous studies have shown that key molecules in CCHF progression seem to be cytokines such as TNF-alpha. In an animal model study, it has been also demonstrated the elevated levels of TNF-alpha concentrations are correlated to disease severity (Ergonul, Tuncbilek et al. 2006, Papa, Bino et al. 2006, Bente, Alimonti et al. 2010).

We have previously demonstrated that CCHFV has the ability to interfere with host Type I IFN response (REF). In **Paper I** we showed that IRF-3 nuclear translocation and induction of ISGs are delayed in CCHFV-infected cells. IRF-3 is a cytoplasmic protein that upon activation is translocated to the nucleus and plays an important role in the induction of IFNs during viral infection. However, it is not known whether this delay indicates that CCHFV is unable to stimulate a strong immune response through RIG-I activation, upstream of IRF-3, or is a result of an unidentified IFN antagonist (Habjan, Andersson et al. 2008).

IRF-3 also seems to play an essential role in mediating induction of apoptotic pathways. In addition to acting as a transcription factor for the induction of antiviral genes, IRF-3 is also involved in cell death by indirect triggering caspase activation (Chattopadhyay, Marques et al. 2010). In **Paper II** we showed that CCHFV has strategies to interplay or interfere with apoptotic molecular signalling pathways through Bax.

It has been reported previously that IRF-3 also has the ability to act as a pro-apoptotic protein, independent of its role as a transcription factor, i.e. that IRF-3 activation pathways make it competent as either a transcription factor or an apoptotic factor (Chattopadhyay, Marques et

al. 2010). Those authors have also shown that the activated IRF-3 induces mitochondrial-dependent apoptosis through Bax, serving as a connection between the innate and the apoptotic response.

As it has been mentioned **Paper I**, CCHFV can delay activation of IRF-3 and can thereby escape the early interferon response but also escape of early activation of IRF3 can delay the initiation of apoptosis in concert with N, which we showed suppresses or delay Bax activity (**Paper III**).

These data together demonstrate that CCHFV has developed strategies to interplay and interfere with apoptosis and the early innate immune response. We believe these results presented in this thesis will shed a light on the molecular mechanism of CCHFV pathogenesis.

In summary, the results presented in **Papers I to IV** have revealed a complex interplay between CCHFV and the regulation of apoptosis in infected cells. In early infection, CCHFV seems to down-regulate certain apoptotic signalling leading to an anti-apoptotic stage. Interestingly, the CCHFV N protein plays a role in this down-regulation while itself could be cleaved by the activated caspase 3 because of a DEVD motif, which is buried in the superhelical form of N but becomes exposed upon conformational change resulting from the binding of short RNAs. However, in late infection, the extrinsic apoptotic pathway becomes activated probably due to the induction of TNF-α and other cytokines by CCHFV infected cells. To this end, we have yet been able to understand how the balance of apoptosis and cleavage of N impact on CCHFV replication and pathogenesis. Further work is therefore needed to map the network of viral-host interactions in CCHFV infection and to define the importance of such interactions for the regulation of innate immune response and induction of viral pathogenesis in suitable animal models.

CONCLUSIONS

In this thesis we

- Demonstrated that replicating CCHFV has the ability to delay the early Type I IFN response in host-probably by intefering with IRF-3 pathway (paper I).

- Found by examination of the primary structure of CCHFV N a potential motif for caspase cleavage (DEVD) at position 266-269 of the nucleocapsid (N) protein. Comparison with available sequences of S segment of CCHFV in the gene bank revealed that this motif was conserved in all available strainst. The cleavage process of N seems to be caspase-3-dependent and caspase activation do not favour the production of CCHF progeny virus (paper II).

- We demonstrated that the conserved caspase-3 cleavage site is located at the top, α12-α13 loop, of the stalk domain. Our results suggest the interaction between N and RNA regulates the conformation of N and determines if the DEVD motif is exposed for caspase cleavage (paper III).

- Demonstrated that caspase activation are supressed early during CCHFV relication cycle. Caspase activation are regulated at mitochondria level early during CCHF infection. At late post CCHF infection apoptosis are most probably induced by pro-inflammatory factors, which in turn activates the extrinsic apoptotic pathway and that pro-inflammatory mediators might induce extrinsic pathways as a secondary effect (paper IV)

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

1) Karlberg H, Sharifi-Mood B, Mousavi-Jazi M, Dilcher M, Lindegren G, Mardani M, Bereskly S, Weidmann M, Mirazimi A.
Molecular and serological findings in suspected patients with Crimean-congo hemrhagic fever Virus in Iran
In press; JMV 2014

2) Vanhomwegen J, Alves MJ, Zupanc TA, Bino S, Chinikar S, Karlberg H, Korukluoğlu G, Korva M, Mardani M, Mirazimi A, Mousavi M, Papa A, Saksida A, Sharifi-Mood B, Sidira P, Tsergouli K, Wölfel R, Zeller H, Dubois P.
Diagnostic assays for Crimean-Congo hemorrhagic Fever
Emerg Infect Dis. 2012 Dec;18(12):1958-65

3) Mousavi-Jazi M, Karlberg H, Papa A, Christova I, Mirazimi A
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Vaccine. 2012 Sep 28;30(44):6225-9

4) Connolly-Andersen AM, Moll G, Andersson C, Akerström S, Karlberg H, Douagi I, Mirazimi A.
Crimean-Congo hemorrhagic fever virus activates endothelial cells.
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5) Oh HL, Akerström S, Shen S, Bereczky S, Karlberg H, Klingström J, Lal SK, Mirazimi A, Tan YJ.
An antibody against a novel and conserved epitope in the hemagglutinin 1 subunit neutralizes numerous H5N1 influenza viruses. J Virol. 2010 Aug;84(16):8275-86. doi: 10.1128/JVI.02593-09

6) Karlberg H, Lindegren G, Mirazimi A
Comparison of antiviral activity of recombinant and natural interferons against crimean-congo hemorrhagic Fever virus.
Open Virol J. 2010 Apr 22;4:38-41

7) Bereczky S, Lindegren G, Karlberg H, Akerström S, Klingström J, Mirazimi A.
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